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Insulin-independent role of adiponectin receptor signaling in *Drosophila* germline stem cell maintenance

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ABSTRACT

Adipocytes have key endocrine roles, mediated in large part by secreted protein hormones termed adipokines. The adipokine adiponectin is well known for its role in sensitizing peripheral tissues to insulin, and several lines of evidence suggest that adiponectin might also modulate stem cells/precursors. It remains unclear, however, how adiponectin signaling controls stem cells and whether this role is secondary to its insulin-sensitizing effects or distinct. *Drosophila* adipocytes also function as an endocrine organ and, although no obvious adiponectin homolog has been identified, *Drosophila AdipoR* encodes a well-conserved homolog of mammalian adiponectin receptors. Here, we generate a null *AdipoR* allele and use clonal analysis to demonstrate an intrinsic requirement for *AdipoR* in germline stem cell (GSC) maintenance in the *Drosophila* ovary. *AdipoR* null GSCs are not fully responsive to bone morphogenetic protein ligands from the niche and have a slight reduction in E-cadherin levels at the GSC-niche junction. Conversely, germline-specific overexpression of *AdipoR* inhibits natural GSC loss, suggesting that reduction in adiponectin signaling might contribute to the normal decline in GSC numbers observed over time in wild-type females. Surprisingly, *AdipoR* is not required for insulin sensitization of the germline, leading us to speculate that insulin sensitization is a more recently acquired function than stem cell regulation in the evolutionary history of adiponectin signaling. Our findings establish *Drosophila* female GSCs as a new system for future studies addressing the molecular mechanisms whereby adiponectin receptor signaling modulates stem cell fate.

Introduction

Adipocytes comprise the largest endocrine organ in the body and actively contribute to energy homeostasis (reviewed in Kershaw and Flier, 2004; Rosen and Spiegelman, 2014). Not surprisingly, dysfunction of adipocytes as a result of obesity or lipodystrophy disrupts the function of other organs, increasing the risk of heart disease, stroke, diabetes, and cancers (Kizer et al., 2011; reviewed in Eckel et al., 2010; Trujillo and Scherer, 2006; Vucenik and Stains, 2012). Adipokines, which are adipocyte-secreted protein hormones, play a major role in mediating adipocyte effects on multiple tissues (reviewed in Cao, 2014). Leptin, for example, signals satiety to the brain and increases metabolism (reviewed in Bluher, 2014), and mutations in leptin or its receptor cause obesity and metabolic defects in mice and humans (Clement et al., 1998; Kakar et al., 2013; Lee et al., 1996; Zhang et al., 1994; Zhao et al., 2014). Adiponectin has a well-known role in sensitizing peripheral tissues to insulin, and activation of adiponectin receptors is thought to increase insulin sensitivity through cell autonomous mechanisms (reviewed in Yamauchi and Kadowaki, 2013). Adiponectin plasma levels are reduced in obese, insulin resistant, or diabetic individuals (reviewed in Yamauchi and Kadowaki, 2013). Adiponectin-deficient mice are mildly glucose intolerant and insulin-resistant (Kubota et al., 2002), and elevated serum expression of adiponectin is protective against diabetes in mice (Combs et al., 2004).

Adipocytes, together with hepatocyte-like oenocytes, constitute an endocrine organ termed the fat body in *Drosophila* (Liu et al., 2009), and recent studies have shown conservation of adipokine signaling modules in this organism (Kwak et al., 2013; Rajan and Perrimon, 2012). For example, the cytokine Unpaired 2 (Upd2) is produced in adipocytes and acts on insulin-producing cells in the brain to control the secretion of insulin-like peptides (ILPs), and *upd2* mutant defects can be rescued by human leptin, despite a lack of primary sequence homology between the proteins (Rajan and Perrimon, 2012). Similarly, *Drosophila* has no obvious adiponectin homolog, but AdipoR, the homolog of mammalian adiponectin receptors, was reported to act in insulin-producing cells to stimulate ILP secretion and control larval metabolism, and to mediate the effects of human adiponectin in *ex vivo* brain cultures (Kwak et al., 2013).

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Despite the considerable focus on the insulin-sensitizing role of mammalian adiponectin in influencing metabolism, several lines of evidence suggest that adiponectin might also control stem cells. For instance, adiponectin induces the proliferation of muscle satellite cells (Fiaschi et al., 2009), hematopoietic stem cells, and adult hippocampal progenitors (DiMascio et al., 2007); increases endothelial progenitor numbers (Shibata et al., 2008); and stimulates regeneration of muscles and other tissues (reviewed in Fiaschi et al., 2014). Conversely, adiponectin deficiency in mice leads to delayed liver regeneration, impaired recovery from renal damage, and delayed wound healing (reviewed in Fiaschi et al., 2014). It remains unclear, however, whether the functions of adiponectin signaling in insulin sensitization and precursor/stem cell regulation are widely conserved, or whether the role of adiponectin in stem cells relates to its effects on insulin sensitivity or is entirely distinct.

The *Drosophila* ovary is a powerful system for research on adult stem cell biology and its connection to whole-body physiology. The ovary is composed of ovarioles, which are strands of chronologically arrayed follicles (Fig. 1A). Each follicle represents a germline cyst encapsulated by follicle cells and is formed from stem cells in the anterior germarium (Fig. 1B). Germline stem cells (GSCs) reside in a specialized niche, composed primarily of cap cells. GSCs are physically attached to cap cells via E-cadherin, and they also receive signals from the niche, including bone morphogenetic protein (BMP) ligands, which are required for maintenance of the stem cell fate (reviewed in Chen et al., 2011). Diet and insulin signaling also control GSCs and their differentiating progeny. On a rich diet, GSCs are well maintained, and GSCs and their progeny proliferate and grow faster than on a poor diet, with multiple diet-dependent factors mediating this response (reviewed in Ables et al., 2012). For example, ILPs directly stimulate the germline to control GSC proliferation, cyst growth, and progression through vitellogenesis (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). In addition, ILPs act on niche cells to indirectly promote GSC maintenance (Hsu and Drummond-Barbosa, 2009). No studies, however, have yet examined the role of adiponectin signaling in GSCs or any other *Drosophila* stem cell type.

In this study, we demonstrate a cell-autonomous, insulin-independent requirement for *AdipoR* in GSC maintenance in the *Drosophila* ovary through genetic mosaic analysis of a newly generated null *AdipoR* allele. *AdipoR* null GSCs are not fully responsive to BMP ligands and have a slight reduction in E-cadherin levels at the GSC-cap cell junction. Conversely, germline-specific overexpression of *AdipoR* inhibits natural GSC loss, suggesting that reduction in adiponectin signaling contributes to the normal decline in GSC numbers observed over time in wild-type females. Surprisingly, we also found that *AdipoR* is not required for insulin sensitization of the germline, as GSC proliferation, cyst growth, and vitellogenesis remain unaffected upon loss of *AdipoR* function. Our findings establish *Drosophila* female GSCs as a new system for future studies addressing the molecular mechanisms whereby adiponectin receptor signaling modulates stem cell fate. Finally, we speculate that stem cell regulation might represent a distinct and evolutionarily more ancient function of adiponectin receptors relative to their role in insulin sensitization.

Materials and methods

Drosophila strains and culture conditions

Fly stocks were maintained on standard cornmeal/molasses/yeast/agar medium at 22–25 °C. For experiments, females (in the presence of wild-type males) were transferred daily onto either standard medium supplemented with wet yeast paste ("rich diet")

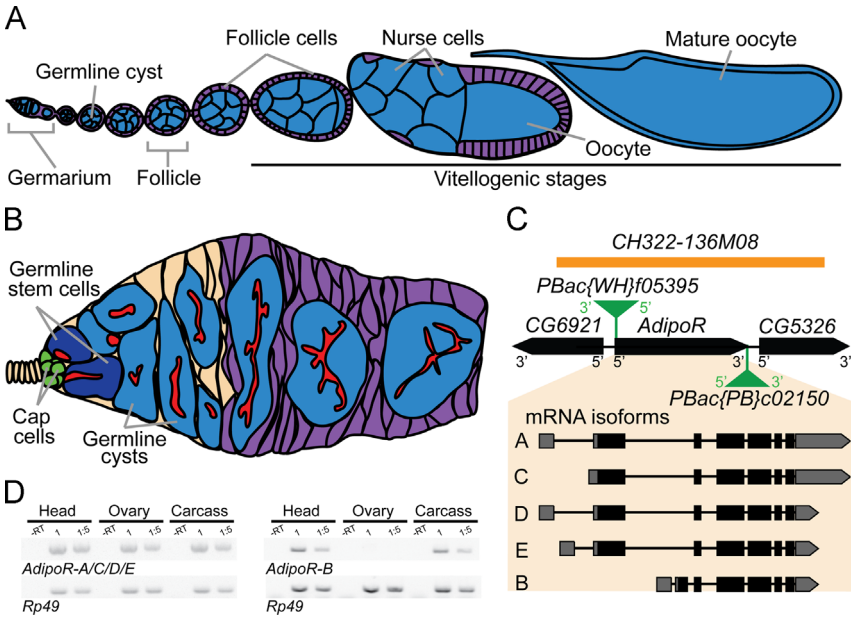

Fig. 1. Generation of a null *AdipoR* allele for the study of its role in *Drosophila* oogenesis. (A) Each *Drosophila* ovariole is composed of a germarium followed by a strand of follicles containing a germline cyst (one oocyte and 15 nurse cells) surrounded by follicle cells. (B) The germarium houses a GSC population (dark blue) juxtaposed to somatic cap cells (green), a key component of the niche. GSCs divide asymmetrically to yield a GSC and a cystoblast, which forms 2-, 4-, 8-, and sixteen-cell germline cysts (light blue) through four rounds of cell division with incomplete cytokinesis. Sixteen-cell cysts are enveloped by follicle cells to form a follicle. GSCs contain an anteriorly anchored fusome (red). The fusome is a germline-specific organelle that adopts stereotypical conformations throughout GSC and germline cyst divisions, allowing the identification of each germ cell stage within the germarium (de Cuevas and Spradling, 1998; Hsu et al., 2008). (C) The *AdipoR* gene encodes five mRNA isoforms corresponding to two distinct protein isoforms (AdipoR-A/C/D/E and Adipo*R-B*). For mRNA isoforms, boxes represent exons, with coding regions colored black. The genomic region is not to scale, and several potential non-coding RNAs have been omitted for simplicity. The entire *AdipoR* gene was cleanly deleted by the recombination of flanking *PiggyBac* transposons (green triangles) to generate a null allele, *AdipoR*^{27}. The *VK37::AdipoR/CH322-136M08* genomic transgene (orange bar) was used for rescue experiments shown in Fig. 4D and Table S2 in Supplementary material. (D) RT-PCR analysis of adult heads, ovaries, and carcasses showing differential expression patterns of *AdipoR-A/C/D/E* and *AdipoR-B*. *Rp49* is a control. PCR reactions were performed with undiluted or 1:5 dilutions of RT reactions. −RT, negative control with no reverse transcriptase in RT reaction.

or molasses/agar ("poor diet") (Drummond-Barbosa and Spradling, 2001). The *FRT82B AdipoR*^{27} recombinant chromosome and *VK37::AdipoR/CH322-136M08; AdipoR*^{27} fly lines were generated by standard crosses. The germline driver *PBac{GreenEye.nosGal4}Dmel2* (referred to here as *nos-Gal4::VP16*), *PBac{PB}c02150*, *PBac{WH}f05395*, and other genetic elements are described in FlyBase (http://www.flybase.org).

Generation of null AdipoR^{27} *allele*

Flies containing *PBac{PB}c02150* and *PBac{WH}f05395* were used to generate a flippase-mediated deletion of *AdipoR* (*AdipoR*^{27}), as described (Thibault et al., 2004). Primers used to verify recombination events and deletion using PCR are listed in Table S1 in Supplementary material.

Generation of AdipoR transgenic strains for rescue and overexpression analyses

The *AdipoR-A/C/D/E* cDNA was first subcloned from *LD23846* (*Drosophila* Genomics Resource Center, DGRC) into *pUASt* (Brand and Perrimon, 1993), linearized with *EcoRI* and *XhoI*, and subsequently excised using *EcoRI* and *XbaI* prior to subcloning into *pUASpI* (Von Stetina et al., 2008) to generate *pUASpI-AdipoR-A/C/D/E*. The *AdipoR-B* coding region (including 20 bp immediately upstream of the initiation codon) was amplified from *IP14059* (DGRC) (see Table S1 in Supplementary material for primers) and, after sequencing, subcloned into *pUASpI* using *SpeI* and *MluI* to generate *pUASpI-AdipoR-B*. Transgenic lines were generated as described (Rubin and Spradling, 1983) either in house or by BestGene, Inc. (Chino Hills, CA).

For the *AdipoR* genomic rescue construct, BAC clone *CH322-136M08* (Fig. 1C), obtained from BAC PAC Resources (https://bacpac.chori.org), was confirmed by sequencing and sent as a bacterial stab to Genetic Services (www.geneticservices.com) for integration into the *attP* docking site *VK37* on chromosome *2L* using ΦC31 integrase, as described (Venken et al., 2009). The *AdipoR* genomic insertion line is referred to as *VK37::AdipoR/CH322-136M08*, according to the suggested nomenclature (Venken et al., 2009).

Genetic mosaic and overexpression analyses

Females of genotype *hs-FLP/+; FRT82B AdipoR***/FRT82B Ubi-GFP* were generated by standard crosses. (*AdipoR** represents null *AdipoR*^{27} or wild-type alleles of the *AdipoR* gene.) Zero- to 3-d-old females were maintained on dry yeast and heat shocked twice daily at 37 °C for 3 d to induce mitotic recombination (Xu and Rubin, 1993). For GSC maintenance and cyst development and growth assays, flies were kept on a rich diet for 3 d after the final heat shock, then either maintained on a rich diet or shifted to a poor diet for 10 d prior to dissection and processing. *AdipoR*^{27} homozygous clones were identified by the absence of green fluorescent protein (GFP), as detected by antibody staining, and GSCs were identified based on their anterior location and typical fusome morphology (de Cuevas and Spradling, 1998; Hsu et al., 2008). To quantify GSC loss, we analyzed all germaria that contained GFP-negative cystoblasts and/or cysts (derived from GFP-negative GSCs), and calculated the percentage of germaria that no longer contained GFP-negative GSCs (i.e. "GSC loss events"), as described (Ables and Drummond-Barbosa, 2010; Hsu and Drummond-Barbosa, 2009). Early germline cysts were staged according to their fusome morphology (de Cuevas and Spradling, 1998), and later egg chambers were staged based on size, nuclear morphology, and yolk uptake (Spradling, 1993). Follicle growth was qualitatively assessed by comparing follicles containing GFP-negative cysts to neighboring GFP-positive follicles. Progression through vitellogenesis was measured by determining the percentage of germline mosaic ovarioles containing a GFP-negative vitellogenic follicle (stage 8 or later) (Spradling, 1993). Statistical significance was determined over at least two independent trials.

To measure GSC proliferation, flies were maintained on a rich diet for 10 d following the last heat shock, then either switched to a poor diet or maintained on a rich diet for an additional 3 d. EdU incorporation assays were performed as described (Ables and Drummond-Barbosa, 2013). The number of EdU-positive GFP-negative GSCs was calculated as a percentage of the total number of GFP-negative GSCs analyzed over eight independent trials. Statistical significance was determined by Chi-square analysis and Student's *t* test.

For rescue experiments, *UAS-AdipoR-B* and *UAS-AdipoR-A/C/D/E* were individually recombined with *nos-Gal4::VP16*, and subsequently introduced into the *hs-FLP/+; FRT82B AdipoR*^{27}*/FRT82B Ubi-GFP* genotype through standard crosses for genetic mosaic analyses. The *VK37::AdipoR/CH322-136M08* genomic transgene was similarly introduced into *hs-FLP/+; FRT82B AdipoR*^{27}*/FRT82B Ubi-GFP* females for genomic rescue experiments.

For overexpression analyses on a rich diet, *nos-Gal4::VP16/AdipoR-B* and *nos-Gal4::VP16/AdipoR-A/C/D/E* females were raised at 18 °C to minimize transgene expression during development. Newly-eclosed females were maintained at 18 °C on a rich diet with wild-type males for 1 to 2 d, then switched to 29 °C for 10 or 20 d prior to dissections. For overexpression analyses on a poor diet, females were raised at 25 °C. Newly-eclosed females were fed a rich diet with wild-type males for 1 to 2 d, then switched to a poor diet at 29 °C for 10 or 20 d prior to dissections.

Immunofluorescence and microscopy

Adult ovaries were dissected in Grace's Insect Medium (Lonza), teased apart, and fixed for 13 min in 5.3% formaldehyde (Ted Pella) in Grace's. Samples were rinsed and washed four times in 0.1% Triton X-100 (Sigma) in phosphate-buffer saline (PBS), or PBT, and blocked for at least 3 h at room temperature or overnight at 4 °C in 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (NGS; Jackson ImmunoResearch) in PBT unless otherwise noted. Samples were incubated at 4 °C overnight with primary antibodies in blocking solution at the following concentrations: mouse anti-Hts (1B1) (DSHB; 1:10); mouse anti-Orb (6H4) (DSHB; 1:10); mouse anti-Lamin C (LC28.26) (DSHB; 1:100); mouse anti-cleaved Caspase 3 (1:50, Cell Signaling); rabbit anti-Bruno (1:1000) (Sugimura and Lilly, 2006); rabbit anti-GFP (1:2500, Torrey Pines); rabbit anti-Nanos (1:3000) (Hanyu-Nakamura et al., 2004); rabbit monoclonal anti-Smad3 (pMad; EP823Y) (1:100, Abcam); rat anti-E-cadherin (DCAD2) (1:3, DSHB); chicken anti-GFP (1:2000, Abcam); guinea pig anti-A2BP1 (1:1000) (Tastan et al., 2010). For anti-A2BP1 labeling, ovaries were processed as described (Tastan et al., 2010). Ovaries stained with anti-pMad were dissected and fixed in Grace's medium supplemented with 25 mM Na_3VO_4 and 10 mM NaF. After primary antibody incubation, samples were washed for 2 h in PBT and incubated for 2 to 4 h in Alexa Fluor 488-, 568-, or 633-conjugated goat species-specific secondary antibodies (1:200, Invitrogen). Samples were mounted in Vectashield with DAPI (Vector Laboratories). Confocal images were acquired using a Zeiss LSM 700 microscope, analyzed using either Zeiss ZEN 2009 or Axiovision software, and equally and minimally enhanced via histogram using Adobe Photoshop CS4. Quantification of pMad and E-cadherin levels was performed as described (Ables and Drummond-Barbosa, 2013).

Apoptag Red In Situ Apoptosis Detection Kit (Millipore) was used following fixation and prior to antibody staining, as described (Drummond-Barbosa and Spradling, 2001). EdU incorporation assays were performed as described (Ables and Drummond-Barbosa, 2013). Briefly, ovaries were dissected in Grace's medium at room temperature and incubated in 100 μM EdU (Invitrogen) in Grace's medium for 1 h prior to being teased apart, fixed, and stained as above. EdU was detected with AlexaFluor-594 via

Click-It chemistry using manufacturer's instructions (Invitrogen) following secondary antibody incubation.

RT-PCR and qPCR analysis

For RT-PCR analysis, fifteen 0- to 3-d-old *yw* females were cultured overnight on standard medium supplemented with wet yeast paste. Head, ovaries, and carcasses were dissected in RNA-*later* (Ambion) for analysis of endogenous *AdipoR* isoforms. For qPCR analysis, fifty 0- to 1-d-old *yw* females were cultured for one week on standard medium supplemented with wet yeast ("rich diet") or molasses/agar medium ("poor diet"), then dissected in RNA*later*. To avoid potential contributions of stage-specific differences in *AdipoR* expression, vitellogenic egg chambers (which are differentially represented on rich versus poor diets) were removed from ovaries prior to RNA extraction. RNA was extracted from all tissues using RNAqueous-4PCR DNA-free RNA Isolation for RT-PCR kit (Ambion) according to the manufacturer's instructions. cDNA was synthesized using SSRII kit (Ambion) according to manufacturer's instructions and used immediately for PCR using primers listed in Table S1 in Supplementary material. *Rp49* was used as a control for RT-PCR. For qPCR, reactions were performed with SYBER Green Supermix (Bio-Rad), and *Rp49*, *Actin 5 C*, and *α-Tubulin* were used as controls.

Results

Generation of the null AdipoR^{27} allele uncovers an essential role during development

Drosophila have no obvious adiponectin homolog based on primary sequence, but a single, well conserved AdipoR homolog is encoded (see Fig. S1 in Supplementary material). Five *AdipoR* mRNA isoforms are predicted, corresponding to two distinct protein isoforms, AdipoR-A/C/D/E and AdipoR-B (Fig. 1C). Based on RT-PCR analysis, *AdipoR-A/C/D/E* and *AdipoR-B* mRNAs are both expressed in adult females; however, while *AdipoR-A/C/D/E* showed robust ovarian expression, *AdipoR-B* levels were below detection in the ovary (Fig. 1D).

Functional analyses of the *AdipoR* gene in *Drosophila* have been limited by the lack of well-characterized genetic mutations in *AdipoR* (Kwak et al., 2013). Therefore, as a first step to analyze the role of *AdipoR* in the GSC lineage during *Drosophila* oogenesis, we generated a null allele of *AdipoR*, *AdipoR^{27}*, by *FLP/FRT*-mediated recombination between transposable elements flanking the *AdipoR* gene and verified the molecular deletion (Fig. 1C; see Table S1 in Supplementary material). In the *AdipoR^{27}* allele, the transcribed region of *AdipoR* is completely removed, leaving neighboring genes intact. *AdipoR^{27}* homozygotes and *AdipoR^{27}/Df(3R)Excel6273*, *AdipoR^{27}/Df(3R)ED6090*, *AdipoR^{27}/Df(3R)ED6093*, or *AdipoR^{27}/Df(3R)ED6085* hemizygotes die during development, and a single copy of *VK37::AdipoR/CH322-136M08*, an *AdipoR* genomic rescue construct (Fig. 1C), completely rescues this lethality (see Table S2 in Supplementary material). Thus, *AdipoR* has an essential role during *Drosophila* development, precluding the analysis of ovaries from homozygous *AdipoR^{27}* females.

AdipoR is not required for GSC proliferation

Insulin signaling cell autonomously promotes GSC proliferation, germline cyst growth, and progression through vitellogenesis during *Drosophila* oogenesis (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). To test if AdipoR signaling sensitizes the germline to stimulation by ILPs, we generated genetic mosaic females and analyzed each of these insulin-dependent processes in homozygous null *AdipoR^{27}* GSC clones (recognized by the absence of a GFP marker) present in the context of neighboring wild-type cells.

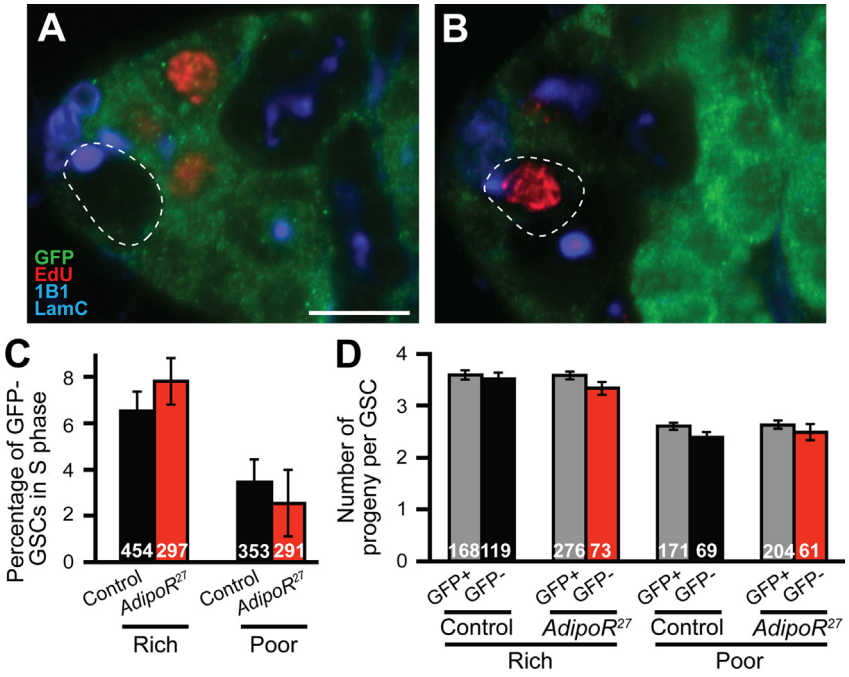

Fig. 2. *AdipoR* is not required cell autonomously for GSC proliferation. (A and B) *AdipoR^{27}* mosaic germaria containing GFP-negative GSCs (dashed lines) without (A) or with (B) EdU incorporation (red). GFP (green) labels wild-type cells; Lamin C (LamC; blue) labels cap cell nuclear envelopes; 1B1 (blue) labels fusomes. Scale bar, 10 μM. (C) Average percentage of GFP-negative GSCs that incorporate EdU in control and *AdipoR^{27}* mosaic germaria. Total number of GFP-negative GSCs analyzed is included inside bars. (D) Average number of cystoblasts and germline cysts per GFP-positive or GFP-negative GSC in control and *AdipoR^{27}* mosaic germaria. Total number of GSCs analyzed is included inside bars. See Fig. 3A for distribution of cystoblast/cyst stages. Error bars represent S.E.M. Differences are not significant by Chi-Square analysis or Student's *t* test.

To examine the requirement for *AdipoR* in GSC proliferation, we first measured the frequency of GFP-negative null *AdipoR*^{27} GSCs in S phase (based on incorporation of the thymidine analog EdU) compared to that of corresponding GFP-negative GSCs in control mosaic females in which all cells are wild-type (Fig. 2A and B) on a rich diet, when high levels of circulating ILPs are available. Comparable frequencies of null *AdipoR*^{27} and control GSCs incorporated EdU (Fig. 2C), indicating similar rates of proliferation. As an independent measure of GSC proliferation, we counted the number of progeny (i.e. cystoblasts and cysts) produced by null *AdipoR*^{27} GFP-negative versus control GFP-positive GSCs within *AdipoR*^{27} mosaic germaria on a rich diet, and found that those numbers were equivalent (Fig. 2D). These results indicate that *AdipoR* is not necessary for ILPs to promote GSC proliferation on a rich diet.

We reasoned that *AdipoR* might be required when nutrients are limiting to enable GSCs to respond effectively to low levels of ILPs. As expected, the fraction of GFP-negative GSCs in S phase was reduced in control mosaic females on a poor diet compared to those on a rich diet (Fig. 2C), in accordance with the known effects of diet on GSC proliferation (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008). The frequency of EdU incorporation in *AdipoR*^{27} GSCs, however, was statistically indistinguishable from that in control GSCs on a poor diet (Fig. 2C). Correspondingly, null *AdipoR*^{27} GFP-negative and control GFP-positive GSCs in *AdipoR*^{27} mosaic germaria yield comparable numbers of progeny on a poor diet (Fig. 2D). We therefore conclude that *AdipoR* is not required for the sensitization of GSCs to either high or low levels of ILPs during their proliferative response to diet.

AdipoR is not required for cyst growth or vitellogenesis

Although GSCs do not require *AdipoR* for their proliferation, stem cells and their progeny can differ in their requirements during the ovarian response to diet (LaFever et al., 2010). Therefore, we directly tested whether *AdipoR* might modulate ILP-sensitivity of the differentiating progeny of GSCs during cyst division, growth, or progression through vitellogenesis. GFP-negative *AdipoR*^{27} cystoblasts, and two-, four-, eight-, and 16-cell cysts were as equally well represented as GFP-positive control cystoblasts and cysts in the same germaria, or as GFP-negative wild-type cystoblasts and cysts in control mosaic germaria on either rich or poor diets (Fig. 3A). These results indicate that early germline divisions were unperturbed by loss of *AdipoR*. Likewise, *AdipoR* was not required for cyst growth because GFP-negative germline cysts within follicles in *AdipoR*^{27} mosaic ovarioles developed at normal rates relative to neighboring GFP-positive cysts and to control GFP-negative cysts regardless of diet (Fig. 3B; 40 GFP-negative *AdipoR*^{27} cysts and 32 GFP-negative control cysts analyzed). To determine if *AdipoR* is required for vitellogenesis, we measured

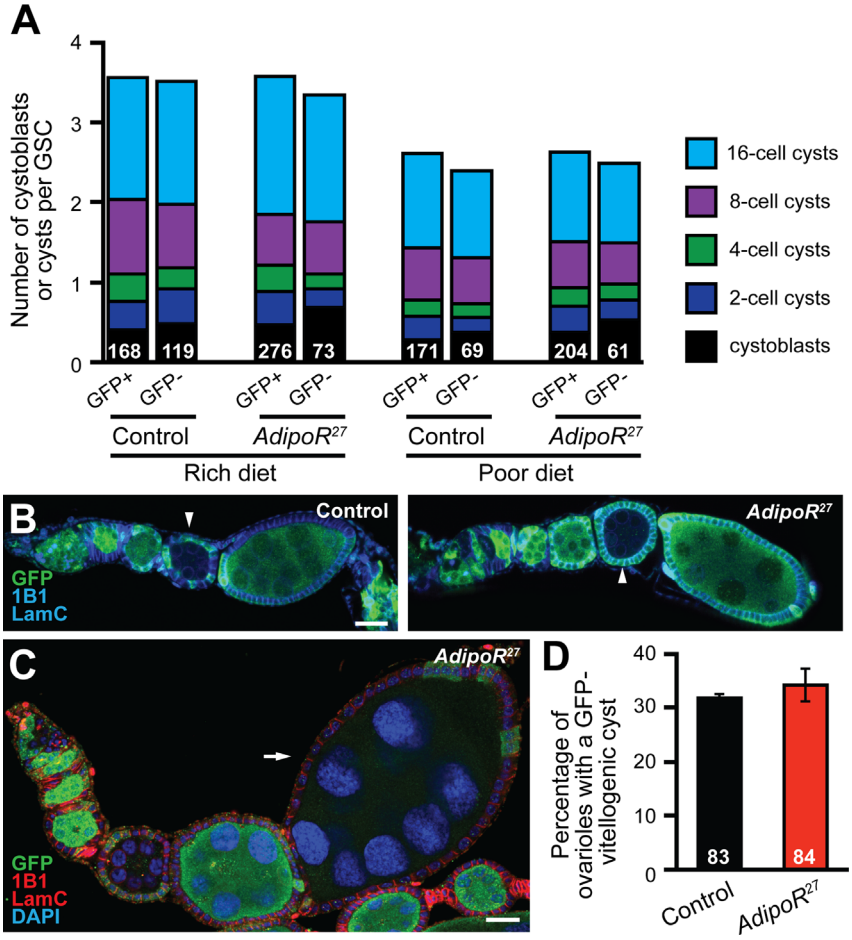

Fig. 3. *AdipoR* is not cell autonomously required for cyst division, growth, or progression through vitellogenesis. (A) Average distribution of cystoblasts, two-, four-, eight-, and 16-cell cysts per GSC in control and *AdipoR*^{27} mosaic germaria maintained on rich or poor diets. The same data are plotted in Fig. 2D. (B) Control (left) and *AdipoR*^{27} (right) mosaic ovarioles displaying GFP-negative germline cysts (arrowheads) that grow normally relative to flanking GFP-positive cysts. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes and follicle cell membranes; LamC (blue) labels cap cell nuclear envelopes. (C) *AdipoR*^{27} mosaic ovariole showing a homozygous mutant GFP-negative germline cyst (arrow) in vitellogenesis. GFP (green) labels wild-type cells; 1B1 (red) labels fusomes and follicle cell membranes; LamC (red) labels cap cell nuclear envelopes; DAPI (blue) labels nuclei. Scale bars, 20 μM. (D) Percentage of control and *AdipoR*^{27} mosaic ovarioles with vitellogenic follicles containing GFP-negative germline cysts. Total numbers of ovarioles analyzed are included inside bars. Error bars represent S.E.M. Differences are not significant by Chi-Square analysis or Student's *t* test.

the frequency of ovarioles containing GFP-negative cysts within vitellogenic follicles in *AdipoR*^{27} compared to control mosaic females (Fig. 3C and D). Over one-third of *AdipoR*^{27} mosaic ovarioles contained at least one GFP-negative vitellogenic germline cyst, comparable to what we observed in control mosaics (Fig. 3D). Together with the GSC proliferation results, these data indicate that *AdipoR* is not required for insulin-dependent processes in the ovarian germline, suggesting that adiponectin signaling does not have a role in insulin sensitization in this context.

AdipoR is cell autonomously required for GSC maintenance

Adiponectin has reported roles in mammalian progenitor cells (reviewed in Fiaschi et al., 2014). It remains unclear, however, whether the control of stem cells by adiponectin signaling is linked to the effect of adiponectin on insulin sensitization. *AdipoR* is clearly not required for GSC proliferation (Fig. 2), an insulin-dependent process (LaFever and Drummond-Barbosa, 2005). We therefore tested if *AdipoR* is cell autonomously required for GSC maintenance, a diet-dependent process that does not require insulin signaling within the germline (Hsu and Drummond-Barbosa, 2009, 2011; Yang et al., 2013).

We measured the occurrence of GSC loss events in control and *AdipoR*^{27} mosaic germaria on both rich and poor diets (Fig. 4A–C). Wild-type GFP-negative GSCs in control mosaics are lost in less than 5% of germaria containing germline clones on a rich diet, and the percentage of control mosaic germaria showing GSC loss increases to 18% on a poor diet, as expected (Fig. 4C) (Hsu and Drummond-Barbosa, 2009). We found that null *AdipoR*^{27} GSCs are lost at significantly higher rates than control GSCs on both diets (Fig. 4C), and these loss rates are comparable to those of previously described GSCs defective for maintenance genes (Ables and Drummond-Barbosa, 2010). GSC loss is fully rescued by *VK37::AdipoR/CH322-136M08*, a genomic *AdipoR* rescue transgene (Figs. 1C, 4D). These results indicate an intrinsic requirement for *AdipoR* in GSC maintenance.

The fold increase in *AdipoR*^{27} GSC loss relative to controls is diet dependent. *AdipoR*^{27} GSCs are lost five times more frequently than control GSCs on a rich diet, whereas *AdipoR*^{27} GSC loss is only two-fold higher than for controls on a poor diet (Fig. 4C). These results imply that a partial reduction in AdipoR signaling contributes to the increase in wild-type GSC loss observed on a poor diet. *AdipoR* mRNA levels, however, are very similar on rich and poor diets (Fig. S2), suggesting that regulation of AdipoR signaling might occur at the level of either AdipoR protein or other upstream or downstream pathway components.

AdipoR is predicted to encode two distinct protein isoforms, AdipoR-A/C/D/E and AdipoR-B (Fig. 1C). To determine whether a specific isoform promotes GSC maintenance, we tested the ability of germline-expressed transgenes encoding each of the isoforms to rescue the *AdipoR*^{27} GSC loss observed in mosaic females. We generated *AdipoR*^{27} genetic mosaic germaria in females expressing *UAS-AdipoR-A/C/D/E* or *UAS-AdipoR-B* transgenes under the control of the germline-specific *nos-Gal4::VP16* driver. Germline expression of either *AdipoR-A/C/D/E* or *AdipoR-B* partially restores *AdipoR*^{27} GSC maintenance (Fig. 4E and F), and the partial rescue likely reflects relative low levels of transgene expression.

AdipoR null GSCs are not lost through apoptosis

We next tested if *AdipoR* promotes GSC maintenance by preventing apoptosis. We examined GFP-negative GSCs in *AdipoR*^{27} and control mosaic germaria for expression of cleaved Caspase 3, an early apoptosis marker, and found no evidence for GSC apoptosis (Fig. 5A and B). Similar results were obtained using ApopTag, a marker of late apoptosis (Fig. 5C and D). Both cleaved Caspase 3 and ApopTag labeled a fraction of later germ cells within both *AdipoR*^{27} and control mosaic germaria (Fig. 5), ruling out technical difficulties with these apoptosis markers. These data suggest that *AdipoR* mutant GSCs are not lost through apoptosis, although we cannot completely rule out alternative cell death mechanisms.

AdipoR is required for robust levels of E-cadherin at the niche-GSC junction and full response of GSCs to BMP ligands

GSCs reside in a specialized niche environment, and both the size of the niche (i.e. number of cap cells) and E-cadherin-mediated GSC-niche adhesion are required for GSC maintenance (Song et al., 2002). We therefore considered the possibility that *AdipoR* function in GSCs might control cap cell number or

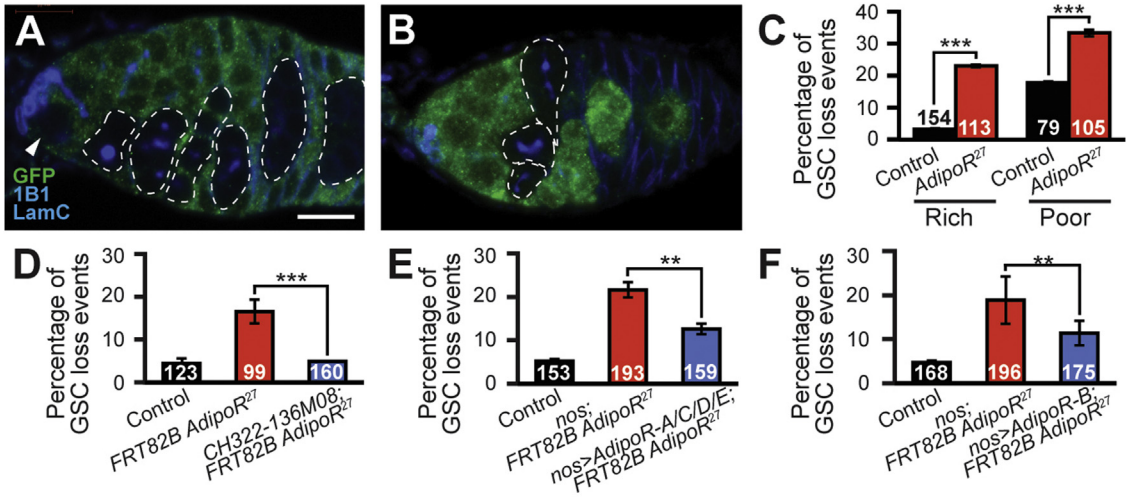

Fig. 4. *AdipoR* is required cell autonomously for GSC maintenance. (A and B) Genetic mosaic germaria showing GFP-negative germline cystoblasts and cysts (outlined) clonally arisen from GFP-negative GSCs (arrowhead) (A). The presence of GFP-negative germline cysts without a GFP-negative GSC indicates a GSC loss event (B). GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes. Scale bar, 10 μM. (C) Quantification of GSC loss events in control and *AdipoR*^{27} mosaic germaria on rich and poor diets showing significant loss of *AdipoR*^{27} GSCs at 13 days after clone induction. (D-F) Graphs showing rescue of *AdipoR*^{27} GSC loss by *VK37::AdipoR/CH322-136M08*, a genomic rescue construct spanning the *AdipoR* gene (see Fig. 1C) (D), or by germline-driven expression of *UAS* transgenes encoding *AdipoR-A/C/D/E* (E) or *AdipoR-B* (F). Total numbers of germaria with mosaic germline counted are included above or inside bars. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, Chi-Square analysis. Error bars represent S.E.M.

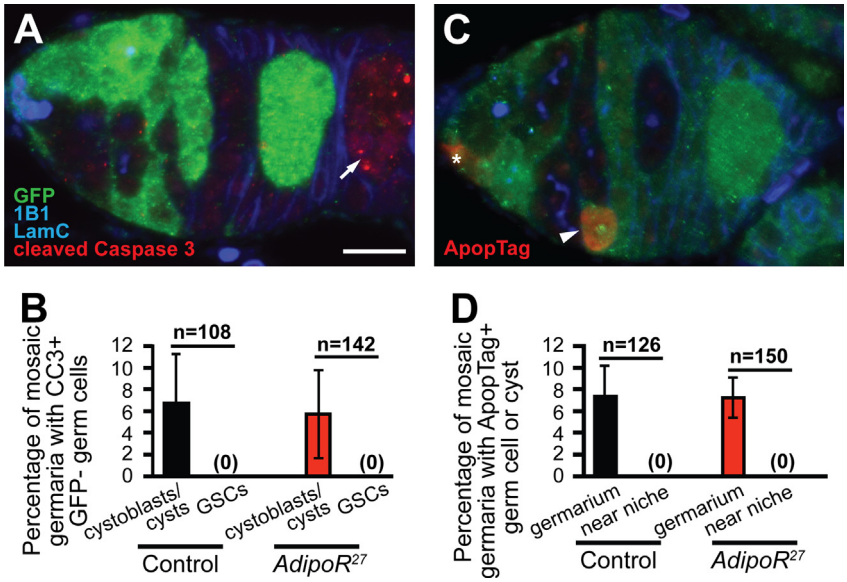

Fig. 5. *AdipoR*^{27} GSCs are not lost through apoptosis. (A) *AdipoR*^{27} mosaic germarium containing a GFP-negative, cleaved Caspase 3 (red)-positive germline cyst (arrow). (B) Percentage of germaria with cleaved Caspase 3-positive, GFP-negative cystoblasts/cysts or GSCs. Total numbers of germaria analyzed are included above. (C) *AdipoR*^{27} mosaic germarium containing a GFP-negative, ApopTag (red)-positive germline cyst (arrowhead) away from the niche. Asterisk indicates an ApopTag-positive somatic cell. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes. Scale bar, 10 μM. (D) Quantification of control and *AdipoR*^{27} mosaic germaria containing ApopTag-positive germ cells. GFP status or specific stage of ApopTag-positive germ cells cannot be reliably scored at this late stage of apoptosis. Total numbers of germline mosaic germaria counted are included above. Differences are not statistically significant by Chi-Square analysis. Error bars represent S.E.M.

E-cadherin levels at GSC-cap cell junctions. Mosaic germaria containing either one or two GFP-negative *AdipoR*^{27} GSCs have comparable cap cell numbers to those with all GFP-positive, control GSCs (Fig. 6A and B). These results indicate that *AdipoR* signaling in GSCs does not have a non-cell autonomous role in controlling niche size or insulin signaling within the niche (which is required for proper cap cell numbers; Hsu and Drummond-Barbosa, 2011). We also measured E-cadherin levels at the junction between cap cells and GFP-negative *AdipoR*^{27} or neighboring control GFP-positive GSCs within mosaic germaria (Fig. 6C and D). Control mosaics showed no difference in E-cadherin intensity between GFP-negative and -positive GSCs (Fig. 6D). In contrast, E-cadherin levels at the cap cell-GSC junction showed a subtle, but statistically significant reduction in *AdipoR*^{27} GSCs relative to neighboring wild-type control GSCs (Fig. 6D). E-cadherin levels vary with the fusome cycle (Hsu and Drummond-Barbosa, 2009); nonetheless, we obtained identical results by restricting our analysis to GSCs with round fusomes (Control: 0.97+0.03, $n=34$ pairs of GFP-negative and neighboring GFP-positive GSCs; *AdipoR*^{27}: 0.85+0.03, $n=65$, $P<0.05$), excluding the possibility of data distortion related to the fusome cycle. We therefore conclude that *AdipoR* has a minor role in GSC-cap cell adhesion.

AdipoR signaling might contribute to GSC maintenance by modulating the ability of GSCs to respond to niche-derived BMP ligands, which repress GSC differentiation (Xie and Spradling, 1998). To test this possibility, we measured the levels of nuclear phosphorylated Mad (pMad), a reporter of BMP signaling (Kai and Spradling, 2003) in GFP-negative relative to GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria (Fig. 6E and F). In control mosaics, GFP-negative and neighboring GFP-positive GSCs have similar pMad levels (Fig. 6F). *AdipoR*^{27} GSCs, however, show a small but significant reduction in pMad levels compared to neighboring GFP-positive GSCs (Fig. 6F). Wild-type pMad levels do not vary with changes in fusome morphology that occur during the cell cycle (GSCs with round fusomes: 26.1+1.36 arbitrary units [a.u.], $n=66$; GSCs with non-round fusome morphologies: 23.3+1.50 a.u., $n=40$, $P=0.16$), ruling out that the difference in pMad levels between control and *AdipoR*^{27} GSCs might be due to sampling bias. These results therefore indicate that *AdipoR* function is required for full receptivity of GSCs to BMP ligands.

The contributions of *AdipoR* to proper E-cadherin and BMP signaling levels in GSCs are modest relative to the robust requirement for *AdipoR* in GSC maintenance, prompting us to examine whether premature expression of differentiation factors in *AdipoR*^{27} GSCs might promote their loss. We compared the expression of Nanos, Bruno, the *Drosophila* homolog of mammalian ataxin 2-binding protein 1 (A2BP1), and Orb in GFP-negative null *AdipoR*^{27} GSCs relative to that in neighboring GFP-positive control GSCs (Fig. 6G–J; 10 to 25 pairs of GSCs analyzed for each marker). Nanos expression in the germarium is highest in mid-stage cysts, with low levels present in GSCs (Forbes and Lehmann, 1998). Nanos expression is unperturbed in *AdipoR*^{27} GSCs (Fig. 6G), indicating that aberrant regulation of Nanos is not responsible for *AdipoR*^{27} GSC loss. We also did not observe precocious expression of germline factors usually restricted to more developed germline cysts in *AdipoR*^{27} GSCs, including the cell cycle regulator Bruno (Sugimura and Lilly, 2006) and the intermediate cyst differentiation marker A2BP1 (Tastan et al., 2010) (Fig. 6H and I). Additionally, Orb expression, usually restricted to the designated oocyte in developing germline cysts (Lantz et al., 1992), appeared normal in *AdipoR*^{27} germline cysts (Fig. 6J). Thus, uncovering the major contribution of *AdipoR* to GSC maintenance will require future studies thoroughly investigating a much wider range of possible molecular mechanisms.

Germline overexpression of AdipoR inhibits GSC loss over time

As *Drosophila* age, GSCs are lost from the niche (Pan et al., 2007; Xie and Spradling, 1998). *AdipoR* is cell autonomously required for GSC maintenance (Fig. 4), and overexpression of adiponectin receptors has been shown to enhance adiponectin signaling in mammals (Chou et al., 2014; Luo et al., 2013). We therefore wondered if increasing the expression of *AdipoR* in the germline might counteract the normal process of GSC loss over time. Indeed, *nanos-Gal4*::*VP16*-driven germline overexpression of either *AdipoR-A/C/D/E* or *AdipoR-B* was sufficient to reverse the GSC loss observed in control Gal4 females on a rich diet (Fig. 7A). On a poor diet, GSC loss was only

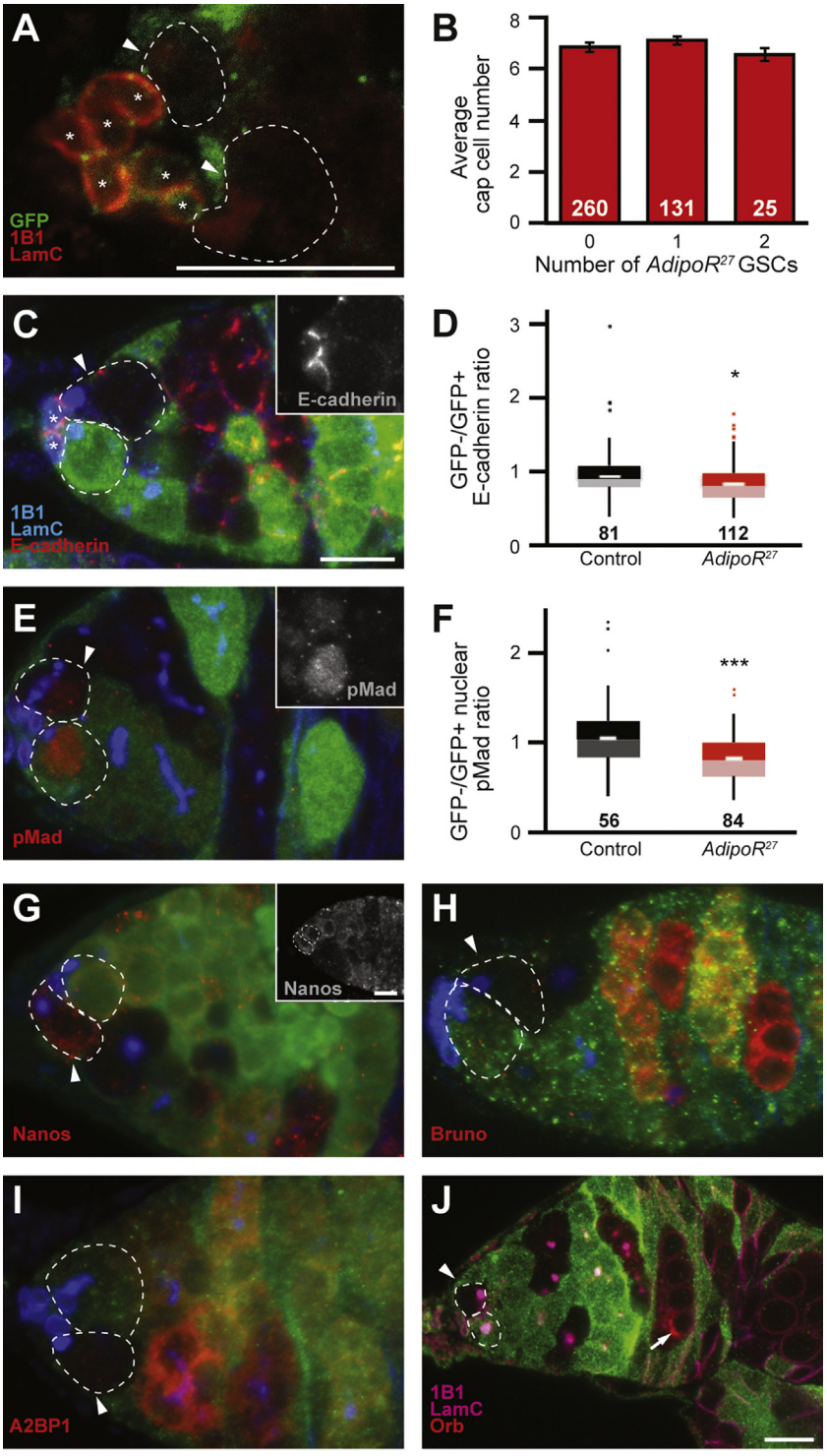

Fig. 6. *AdipoR* is required for full levels of BMP signaling and E-cadherin at the GSC-cap cell junction, but not for proper cap cell numbers or expression of known germline differentiation markers. (A) Mosaic germarium containing two *AdipoR*^{27} null GSCs (GFP-negative) with six visible cap cells (asterisks). (B) Quantification of the number of cap cells in *AdipoR*^{27} mosaic germaria containing zero, one, or two *AdipoR*^{27} GSCs. Number of analyzed germaria are included inside bars. Error bars represent S.E.M. (C) *AdipoR*^{27} null GSCs (GFP-negative) have a subtle, but significant, decrease in E-cadherin (red; greyscale image shown in inset) levels at the junction of GSCs and cap cells (asterisks) compared to neighboring GFP-positive control GSCs. (D) Box-and-whiskers plot showing the ratio of E-cadherin levels at the GSC-cap cell junction for GFP-negative to those for neighboring GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria. (E) Nuclear phosphorylated Mad (pMad, red; greyscale image shown in inset) levels are slightly but significantly reduced in *AdipoR*^{27} null GSCs compared to neighboring GFP-positive controls. (F) Box-and-whiskers plot showing the ratio of nuclear pMad intensity in GFP-negative to that in neighboring GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria. Total numbers of GSC pairs analyzed are included below. White lines indicate averages and points represent outliers outside of the 95% confidence interval. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$, Student's *t* test. (G-J) The expression of Nanos (red; greyscale image shown in inset, G), Bruno (red; H), A2BP1 (red; I), and Orb (red; J) is unperturbed in *AdipoR*^{27} GFP-negative compared to neighboring GFP-positive GSCs in mosaic germaria. GSCs are outlined. Arrowheads indicate GFP-negative *AdipoR*^{27} null GSCs. Arrow indicates normal expression of Orb in GFP-negative *AdipoR*^{27} cyst in region 2B. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes, except in (A) and (J), where 1B1 and LamC are labeled in red and magenta, respectively. Scale bars, 10 μM.

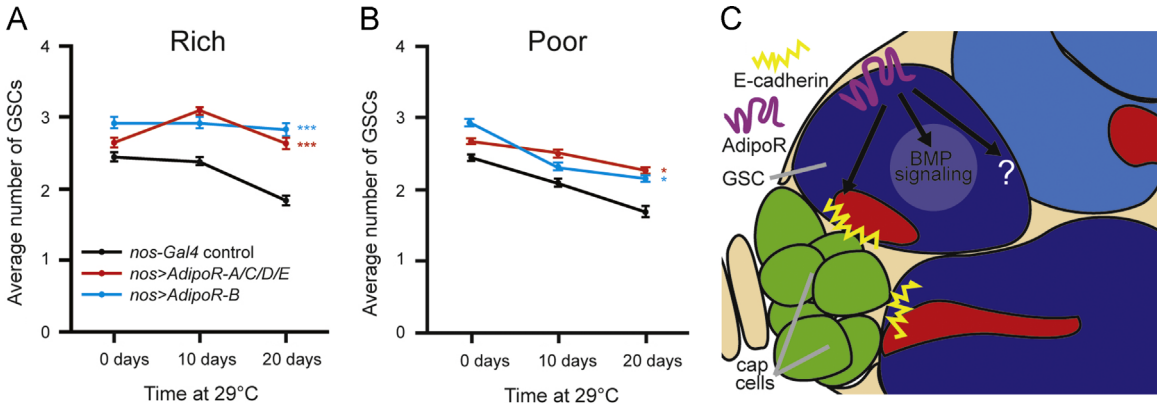

Fig. 7. Germline overexpression of *AdipoR* counteracts normal GSC loss over time. (A) Females expressing either *UAS-AdipoR-A/C/D/E* or *UAS-AdipoR-B* transgenes in the germline driven by *nanos-Gal4::VP16* (*nos-Gal4*) maintain GSCs better than control females carrying *nos-Gal4* alone on a rich diet. Newly eclosed females raised at 18 °C were switched to 29 °C for the indicated number of days. (B) Females expressing *nos-Gal4*-driven *UAS-AdipoR* transgenes maintain GSCs slightly better than *nos-Gal4* control females on a poor diet. Newly eclosed females raised at 25 °C were switched to 29 °C for the indicated number of days. At least 70 (A) or 80 (B) germaria were scored for each time point. Error bars represent S.E.M. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$, Two-way ANOVA with interaction. (C) Model for how *AdipoR* controls GSC maintenance independently of insulin signaling. *AdipoR* acts intrinsically within GSCs to promote GSC maintenance, unequivocally demonstrating that *AdipoR* controls GSCs independently of any direct effects on insulin signaling, which acts instead in cap cells to maintain GSCs (Hsu and Drummond-Barbosa, 2009). Despite its major role in GSC maintenance, the contribution of AdipoR to the modulation of BMP signaling and E-cadherin levels at the GSC-cap cell junction is relatively small, suggesting that additional unknown effectors of AdipoR signaling are likely major players.

partially prevented by overexpression of either AdipoR isoform (Fig. 7B), consistent with the fact that multiple diet-dependent pathways control GSC maintenance (Ables and Drummond-Barbosa, 2010; Ables et al., 2012; Hsu and Drummond-Barbosa, 2009, 2011; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010). These results suggest that a decline in AdipoR signaling may contribute to the normal loss of GSCs that occurs over time.

Discussion

Adiponectin signaling has been reported to control progenitor cells and promote tissue regeneration (Chiarugi and Fiaschi, 2010; DiMascio et al., 2007; Fiaschi et al., 2009, 2014; Shibata et al., 2008), although it had remained unknown whether this role is linked to the insulin-sensitizing effects of adiponectin. In this study, we demonstrate in a highly tractable genetic model organism that adiponectin signaling is intrinsically required for stem cell maintenance independently of insulin-sensitization. *AdipoR* is cell autonomously required for GSC maintenance in the *Drosophila* ovary, presumably in part through enhancement of BMP signaling and E-cadherin-mediated adhesion to the niche, although additional mechanisms are likely involved (Fig. 7C).

Surprisingly, *AdipoR* is not required to sensitize germ cells in the *Drosophila* ovary to ILPs for the control of GSC proliferation, cyst division and growth, and vitellogenesis. Moreover, the cell autonomous role of *AdipoR* in GSC maintenance is clearly independent of insulin signaling, which is instead indirectly required in cap cells to maintain normal GSC numbers in the niche (Hsu and Drummond-Barbosa, 2009, 2011). We therefore speculate that the requirement for *AdipoR* in ovarian GSC maintenance might reflect an ancient role of adiponectin receptors, with insulin sensitization representing a more recently acquired function during their evolution.

The role of *AdipoR* in GSC maintenance appears to partially depend on diet, as the increase in GSC loss relative to controls in response to *AdipoR* loss-of-function on a rich diet is more severe than on a poor diet. A potential mechanism linking *AdipoR* function to diet might be at the level of production of its yet-unidentified ligand(s) in *Drosophila*. Likewise, such ligand(s) may also be regulated with age, given that overexpression of AdipoR isoforms can revert normal GSC loss over time. The *Drosophila* genome does not encode an obvious homolog of adiponectin based on primary sequence, suggesting that the AdipoR ligand(s) has conserved three-dimensional structure in the absence of sequence conservation. In fact, osmotin, a plant ligand for the adiponectin receptor, has less than 10% sequence identity to mammalian adiponectin, but has similar tertiary structure and molecular function (Narasimhan et al., 2005). It will also be important to determine the source, in addition to the identity and regulatory mechanisms, of the AdipoR ligand(s). While adiponectin is the most abundant transcript in human adipocytes (Maeda et al., 1996), there is evidence that adiponectin is not produced strictly in adipocytes (Delaigle et al., 2006; Krause et al., 2008), raising the possibility that the *Drosophila* functional ortholog of adiponectin could be produced in other tissues.

Another focus for future investigation should be how adiponectin receptor signaling regulates stem cell maintenance. *Drosophila AdipoR* function in GSCs provides a small contribution towards full levels of E-cadherin at the niche junction and of BMP signaling. *Lissencephaly-1*, which is intrinsically required for GSC maintenance, also regulates both BMP signaling and E-cadherin levels in ovarian GSCs (Chen et al., 2010). In the *Drosophila* testis, E-cadherin trafficking and BMP signaling are linked (Michel et al., 2011). Aging female GSCs have reduced E-cadherin and BMP signaling levels (Pan et al., 2007), and we find that overexpression of AdipoR isoforms can reverse the normal GSC loss that occurs over time. It will therefore be interesting to determine whether regulation of BMP signaling and E-cadherin downstream of *AdipoR* occur separately or as part of the same signaling axis. Nevertheless, it is unlikely that the slight reductions in BMP signaling and E-cadherin levels measured in *AdipoR* null GSCs fully account for their markedly increased rate of loss. In fact, while BMP signaling controls both the self-renewal and proliferation of GSCs (Xie and Spradling, 1998), AdipoR signaling is not intrinsically required for GSC proliferation, further emphasizing the weakness of the effect of AdipoR on BMP signaling. Future studies addressing additional molecular mechanisms controlling GSC maintenance downstream of AdipoR should yield useful information that is potentially applicable to understanding the role of mammalian adiponectin signaling in precursor cells.

Acknowledgments

K.L. and D.D.B. designed experiments, analyzed and interpreted the data, and wrote the paper; K.L. performed all experiments included in the paper; L.L.S. generated the *pUASpI-AdipoR-A/C/D/E* transgenic line, designed, generated and confirmed the *AdipoR*^{27} allele, and performed initial functional characterization of *AdipoR*^{27} and preliminary analysis of expression of *AdipoR* isoforms. We thank M. Lilly, M. Buszczak, A. Nakamura, and the Developmental Studies Hybridoma Bank for antibodies; the Bloomington Stock Center (supported by NIH P40 OD018537) for *Drosophila* Stocks; and BAC PAC and the *Drosophila* Genomics Resource Center (supported by NIH P40 OD 010949) for genomic and cDNA constructs, respectively. We are grateful to A. Armstrong and S. Matsuoka for critical reading of the manuscript. This work was supported by NIH R01 GM069875 (D.D.B.). K.L. was supported by training grant NIH T32 CA009110.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.12.033.

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